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Screening of *Streptococcus pneumoniae* ABC Transporter Mutants Demonstrates that LivJHMGF, a Branched-Chain Amino Acid ABC Transporter, Is Necessary for Disease Pathogenesis▽

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Bacterial ABC transporters are an important class of transmembrane transporters that have a wide variety of substrates and are important for the virulence of several bacterial pathogens, including *Streptococcus pneumoniae*. However, many *S. pneumoniae* ABC transporters have yet to be investigated for their role in virulence. Using insertional duplication mutagenesis mutants, we investigated the effects on virulence and in vitro growth of disruption of 9 *S. pneumoniae* ABC transporters. Several were partially attenuated in virulence compared to the wild-type parental strain in mouse models of infection. For one ABC transporter, required for full virulence and termed LivJHMGF due to its similarity to branched-chain amino acid (BCAA) transporters, a deletion mutant (Δ*livHMGF*) was constructed to investigate its phenotype in more detail. When tested by competitive infection, the Δ*livHMGF* strain had reduced virulence in models of both pneumonia and septicemia but was fully virulent when tested using noncompetitive experiments. The Δ*livHMGF* strain had no detectable growth defect in defined or complete laboratory media. Recombinant LivJ, the substrate binding component of the LivJHMGF, was shown by both radioactive binding experiments and tryptophan fluorescence spectroscopy to specifically bind to leucine, isoleucine, and valine, confirming that the LivJHMGF substrates are BCAAs. These data demonstrate a previously unsuspected role for BCAA transport during infection for *S. pneumoniae* and provide more evidence that functioning ABC transporters are required for the full virulence of bacterial pathogens.

Bacterial ABC transporters are an important class of transmembrane transporters that are involved in the import and export of a wide variety of substrates, including sugars, amino acids, peptides, polyamines, and cations (11, 12, 17, 39). A typical ABC transporter consists of four membrane-associated proteins consisting of two ATP-binding proteins (ATPases) and two membrane-spanning proteins (permeases) (11, 17). These may be fused in a variety of ways to form multidomain polypeptides, but typically permeases consist of six putative α-helical transmembrane segments that act as a channel through which substrates are transported across the membrane (11, 17). ABC transporters that import their substrate also contain a substrate-binding protein (SBP) that is present in the periplasm of gram-negative bacteria and most often as a lipoprotein bound to the outer surface of the membrane in gram-positive bacteria (17). These SBPs bind to the substrate before it is transferred across the cell membrane and therefore confer substrate specificity for the ABC transporter. Approximately 5% of the *Escherichia coli* and *Bacillus subtilis* genomes encode components of ABC transporters, highlighting the importance of ABC transporters for the physiology of both gram-positive and gram-negative bacteria (12, 30). ABC transporters are known to influence many cellular processes. including antibiotic resistance, nutrient acquisition, adhesion, protein secretion, environmental sensing, spore formation, conjugation, and growth under stress conditions (39). As a consequence, many ABC transporters have been shown by signature-tagged mutagenesis (STM) screens to be important for the virulence of a range of bacterial pathogens, including *Yersinia* spp., *Staphylococcus aureus*, and *Streptococcus pneumoniae* (9, 15, 22, 26), and these data have been supported by publications on the functions of individual ABC transporters (6, 7, 38, 45).

The annotated genome sequence of the TIGR4 strain of the common gram-positive pathogen *S. pneumoniae* contains 73 ABC transporters (4, 13). Several ABC transporters required for substrate uptake have been described in some detail previously, and some of these are known to be important for full virulence, including the cation transporters PsaA, PiuA, PiaA, and PitA (6, 7, 27, 34) and the polyamine transporter PotABCD

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(44). Why disruption of these ABC transporter functions affect virulence is probably due to a variety of mechanisms. These include effects on micronutrient acquisition under stress conditions, such as reduced iron uptake after disruption of PiuA, PiaA, and PitA; increased sensitivity to oxidative stress due to loss of polyamine or manganese uptake by PotABCD or PsaA, respectively (25, 43, 44); and impaired adhesion to cell surfaces related to disruption of PsaA (2, 20). In addition, since intracellular levels of cations and other micronutrients can influence gene regulation (20), impaired uptake of micronutrients could affect bacterial adaptation to the host environment. SBP components of ABC transporters are attached to the external surface of the bacterial membrane, where they are exposed to interactions with the environment, and their sequences are usually highly conserved between different strains of the same bacterial species. As a consequence, SBPs have been investigated as potential protein vaccine candidates, and PiuA, PiaA, PsaA, and PotD have all been shown to be effective vaccines in animal models at preventing *S. pneumoniae* infection (12, 23).

Given the importance of acquisition of various minerals and nutritional substrates for bacterial growth and virulence in vivo, some of the *S. pneumoniae* ABC transporters that have not yet been investigated are also likely to influence the pathogenesis of *S. pneumoniae* infections. Using mouse models of infection, we have therefore assessed the potential role during virulence of nine *S. pneumoniae* ABC transporters that have not, as far as we are aware, previously been investigated. Several ABC transporters were identified as required for full *S. pneumoniae* virulence in models of septicemia or pneumonia. The function of one of these ABC transporters, termed *livJHMGF*, which BLAST searches suggest is a member of the hydrophobic amino acid transporter subfamily and is likely to be a branched-chain amino acid (BCAA) transporter (40), was investigated in more detail.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *E. coli* strains DH5α, Novablue competent cells (Novagen), JM109 (Promega), and M15 (Qiagen) were used for cloning procedures. The capsular serotype 3 *S. pneumoniae* strain 0100993, originally isolated from a patient with pneumonia and obtained from SmithKline Beecham, Plc. (22), was used to construct *S. pneumoniae* mutant strains for the majority of the in vitro and in vivo phenotype analysis, with additional experiments performed using the capsular serotype 2 strain D39 and the serotype 4 strain TIGR4. *E. coli* was cultured at 37°C using Luria-Bertani broth or agar plates, and *S. pneumoniae* strains were cultured in the presence of 5% CO_2 at 37°C on Columbia agar (Oxoid) supplemented with 5% horse blood (TCS Biosciences) or in Todd-Hewitt broth supplemented with 0.5% yeast-extract (Oxoid) or CDEM medium (42). Plasmids and mutant strains were selected for using appropriate antibiotics (10 μg of chloramphenicol, 100 μg of carbenicillin, and 25 μg of kanamycin ml^{-1} for *E. coli* and 10 μg of chloramphenicol and 0.2 μg of erythromycin ml^{-1} for *S. pneumoniae*). Stocks of *S. pneumoniae* were stored as single-use 0.5-ml aliquots of THY broth culture (optical density at 580 nm [OD_{580}] of 0.3 to 0.4) at −70°C in 10% glycerol. The growth of *S. pneumoniae* strains in broth was monitored by measuring the OD_{580}.

Nucleic acid manipulations and RT-PCR. *S. pneumoniae* genomic DNA was extracted from bacteria grown in THY by using a modified Wizard genomic DNA kit (Promega), and RNA was extracted by using an SV total RNA extraction kit (Promega) as previously described (6). Restriction digests, ligation of DNA fragments, fractionation of DNA fragments by electrophoresis, and transformation of *E. coli* (by heat shock) were performed according to established protocols (33). DNA fragments were purified from electrophoresis gels by using a QIAquick gel extraction kit. Reverse transcriptase PCR (RT-PCR) was performed by using the Access RT-PCR system (Promega) and gene-specific primers (see Table 2). The National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast) was used to perform BLAST searches and alignments of the available complete and incomplete bacterial nucleotide and protein databases. Primers for both PCR and RT-PCR were designed using sequences displayed by the Artemis software.

Construction of *S. pneumoniae* mutant strains. Strains and plasmids used for the present study are described in Table 1, and the primers used for PCR and sequencing are listed in Table 2. Strains containing disrupted copies of genes encoding ABC transporters were constructed by using insertional duplication mutagenesis (IDM) and the plasmid pID701 (22). Internal fragments of target genes were amplified by PCR from *S. pneumoniae* genomic DNA using primers designed from the TIGR4 genome sequence (Table 2) (http://www.tigr.org) (40). Amplified products were ligated into the XbaI site of pID701 and transformed into *E. coli* for amplification of plasmid DNA. Plasmids with the correct inserts were then used to transform *S. pneumoniae* strain 0100993 using competence stimulating peptide 1 (kindly provided by D. Morrison) and selection with chloramphenicol according to established protocols (6, 22).

The Δ*livHMGF* strain was constructed by overlap extension PCR (36) using a transformation fragment in which a contiguous fragment of *S. pneumoniae* genomic DNA from Sp0749 to Sp0754 had the genes *livHMGF* (Sp0750 to Sp0753) replaced by the erythromycin resistance cassette *ery*. Two products corresponding to 883 kb 5′ (primers Sp0749F and Ery-Sp0749R) and 833 kb 3′ (primers Ery-Sp0753F and Sp0754R) to *livHMGF* were amplified from *S. pneumoniae* genomic DNA by PCR carrying 3′ and 5′ linkers complementary to the 5′ and 3′ portion of the *ery* gene, respectively. *ery* was amplified from pACH74 using PCR and the primers EryF and EryR (21). Reaction conditions for amplifying these three fragments were as follows: a volume of 200 μl containing 100 pmol of primers, 200 μM deoxynucleoside triphosphates (dNTPs; Bioline), and 0.5 U of *Taq* DNA polymerase (Sigma) using an initial denaturing step at 94°C for 4 min, followed by a PCR cycle of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min for 30 cycles, with a final extension at 72°C for 10 min. Cleaned individual PCR products were fused by using a two-step PCR, with an initial PCR to fuse the 5′-flanking DNA fragment with *ery*, followed by addition of the 3′-flanking DNA. The conditions for the first PCR were as follows: reaction volume of 20 μl containing 8.7 μl of nuclease free water, 1 μl of buffer, 1 μl of 2 mM dNTPs, 0.4 μl of 50 mM $MgSO_4$, 0.2 μl of *Taq* polymerase (Bioline), and approximately 50 ng of each PCR product with no primers and an initial denaturing step at 94°C for 2 min, followed by 10 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 1 min. The conditions for the second PCR were as follows: a reaction volume of 100 μl containing 68.2 μl of nuclease-free water, 10 μl of buffer, 10 μl of 2 mM concentrations of dNTPs, 4 μl of 50 mM $MgSO_4$, 100 pmol of primers Sp0749F and Sp0754R, 3 μl of unpurified PCR product from the first PCR, and 0.8 μl of *Taq* polymerase and an initial denaturing step at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 2.5 min, with a final extension at 72°C for 3 min. The fusion PCR products were then analyzed on a 1% agarose gel, and the desired DNA band was excised and purified by using Qiagen QIAquick columns and transformed into the *S. pneumoniae* 0100993, D39, and TIGR4 strains as described above (7, 22). Plasmid and *S. pneumoniae* mutant identities were confirmed by PCR using insert (Table 2) and plasmid-specific primers (Sp1 and Sp3), followed by sequencing of the PCR products (performed by Lark Technologies, Inc. [United Kingdom] or UCL Sequencing Services using the BigDye terminator technique and gene-specific PCR primers).

Expression and characterization of His_6-LivJ lipoprotein. The lipoprotein LivJ (Sp0749) was expressed in *E. coli* and purified by using an N-terminal His-tagged QIAexpressionist system (Qiagen). Primers Sp0749Fwd and Sp0749Rv amplified a full-length *livJ* (excluding the 5′ portion encoding the predicted N-terminal signal peptide), which was ligated into the pQE30 expression vector to make the plasmid pPC139 and transformed into *E. coli* strain M15. Protein expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) and native His_6-LivJ purified using Ni-NTA affinity columns according to the QIAexpressionist manual. Purification products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shown to contain a ≥95% purity of protein of the expected size for His_6-LivJ, and the identity of the recombinant protein was confirmed by peptide mass fingerprinting as reported previously (35).

[^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]maltose uptake and binding assays. Radioactive uptake assays were performed by the rapid filtration method as previously described (45) with minor modifications. *S. pneumoniae* strains were grown in THY medium until the OD_{620} reached 0.2 to 0.4. These experiments were performed using a capsular serotype 2 *S. pneumoniae* strain (D39) since the mucoid colonies of the capsular serotype 3 strain prevented effective pelleting of the bacteria for these assays. Bacteria were harvested at 13,000 × *g* for 20 min and resuspended in 50 mM potassium phosphate buffer (pH 7.2) with 1 mM

TABLE 1. Strains and plasmids constructed and/or used in this study

Plasmid or strain	Description (source or reference)[a]
Plasmids	
pID701	Shuttle vector for IDM transformation of *S. pneumoniae*: Cm^r (22)
pPC110	pID701 containing an internal portion of Sp0090 in the XbaI site: Cm^r (this study)
pPC111	pID701 containing an internal portion of Sp0149 in the XbaI site: Cm^r (this study)
pPC112	pID701 containing an internal portion of Sp0610 in the XbaI site: Cm^r (this study)
pPC113	pID701 containing an internal portion of Sp0710 in the XbaI site: Cm^r (this study)
pPC114	pID701 containing an internal portion of Sp1796 in the XbaI site: Cm^r (this study)
pPC115	pID701 containing an internal portion of Sp1824 in the XbaI site: Cm^r (this study)
pPC116	pID701 containing an internal portion of Sp0750 in the XbaI site: Cm^r (this study)
pPC117	pID701 containing an internal portion of Sp1690 in the XbaI site: Cm^r (this study)
pPC118	pID701 containing an internal portion of Sp0846 in the XbaI site: Cm^r (this study)
pPC119	pID701 containing an internal portion of Sp2084 in the XbaI site: Cm^r (this study)
pPC120	pID701 containing an internal portion of Sp2108 in the XbaI site: Cm^r (this study)
pPC139	pQE30 carrying full length *livJ*: Km^r Amp^r (this study)
Strains	
0100993	*S. pneumoniae* capsular serotype 3 clinical isolate (22)
ΔSp0090	0100993 containing an insertion made with plasmid pPC110: Cm^r (this study)
ΔSp0149	0100993 containing an insertion made with plasmid pPC111: Cm^r (this study)
ΔSp0610	0100993 containing an insertion made with plasmid pPC112: Cm^r (this study)
ΔSp0750	0100993 containing an insertion made with plasmid pPC116: Cm^r (this study)
ΔSp0846	0100993 containing an insertion made with plasmid pPC118: Cm^r (this study)
ΔSp1690	0100993 containing an insertion made with plasmid pPC117: Cm^r (this study)
ΔSp1796	0100993 containing an insertion made with plasmid pPC114: Cm^r (this study)
ΔSp1824	0100993 containing an insertion made with plasmid pPC115: Cm^r (this study)
ΔSp2084	0100993 containing an insertion made with plasmid pPC119: Cm^r (this study)
ΔSp2108	0100993 containing an insertion made with plasmid pPC120: Cm^r (this study)
Δ*livHMGF*	0100993 containing the *livHMGF* deletion construct: Erm^r (this study)
T4Δ*livHMGF*	TIGR4 containing the *livHMGF* deletion construct: Erm^r (this study)

[a] Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Erm^r, erythromycin resistance.

$MgCl_2$ to an OD_{620} between 0.8 and 1.1. The uptakes of leucine, isoleucine, and maltose were determined in 1-ml assays containing 0.85 ml of bacterial cells and a final concentration of 25 μM maltose, isoleucine, or leucine containing 0.125 μCi of ^{14}C-labeled substrate ([^{14}C]isoleucine, [^{14}C]leucine, and [^{14}C]maltose; GE Healthcare, United Kingdom). Samples (150 μl) containing bacteria and radioactive and nonradioactive substrates were removed at various time intervals (0, 1, 2, and 3 min) and immediately filtered through glass fiber filters (Whatman GF/F) and washed twice with 50 mM potassium phosphate buffer. The washed filters were placed in scintillation vials in 5 ml of Ready-Safe scintillation cocktail (Beckman Coulter), and the radioactivity was determined by using a Wallac 1214 RackBeta liquid scintillation counter. A bicinchoninic acid protein assay (Sigma Aldrich, United Kingdom) determined that an OD_{620} of 1 was equivalent to 0.238 mg of protein, and this figure was used to convert the radioactivity counts to nmol of solute per mg of protein.

For radioactive substrate binding assays, 100 μl of purified His_6-LivJ (27.5 μg) was incubated with a 5 μM substrate containing 0.1 μCi of ^{14}C-labeled ligand (GE Healthcare) and further incubated on ice for 10 min. To this mixture, 1 ml of saturated ammonium sulfate was added, followed by incubation on ice for 20 min, and then filtered onto glass fiber filter papers (GF/F; Whatman). The filters were then washed with 4 ml of saturated ammonium sulfate and dried for 5 min. The filters were allowed to equilibrate in Ready-Safe scintillation cocktail for 20 min, and the radioactivity was determined by using a Wallac 1214 RackBeta liquid scintillation counter (31).

Tryptophan fluorescence spectroscopy. Purified His_6-LivJ was used for tryptophan fluorescence spectroscopy using a Hitachi F-2500 spectrofluorimeter at an excitation wavelength of 280 nm (slit width, 3 nm) and an emission wavelength of 309 nm (slit width, 3 nm) (41). The assay was performed by adding test amino acids (Sigma) dissolved in 10 mM NaH_2PO_4 to 0.5 μM His_6-LivJ in 1.5 ml of 50 mM Tris-HCl (pH 8) in a sample cuvette maintained at 25°C in the spectrofluorimeter with continuous stirring. The slit width of the spectrofluorimeter was adjusted to reduce photobleaching of the protein (41).

Animal models of infection. Infection experiments were performed in age- and sex-matched groups of outbred CD1 mice (Charles River Breeders) between 4 to 8 weeks old. For mixed infections, equivalent numbers of bacteria from stocks of wild-type and mutant *S. pneumoniae* strains were mixed and diluted to the appropriate concentration. For the nasopharyneal colonization model, 10^7 CFU of bacteria in 10 μl were administered by intranasal (i.n.) inoculation under halothane general anesthesia, and nasal washes were obtained after 2 days. For the systemic model of infection, 10^3 CFU of bacteria in 100 μl were inoculated by intraperitoneal (i.p.) injection, and spleen homogenates were obtained at 24 h (6, 21). For the pneumonia model, 5×10^6 CFU of bacteria in 40 μl were given by i.n. inoculation under halothane general anesthesia, and lung and spleen homogenates were obtained at 48 h (8, 21). Aliquots from samples recovered from mice were plated on plain and Cm containing plates to allow calculation of a competitive index (CI). The CI was calculated as follows: the ratio of mutant to wild-type strain recovered from mice divided by the ratio of mutant to wild-type strain in the inoculum (5). A CI of <1 indicates that the mutant strain is attenuated in virulence compared to the wild-type strain, and the lower the CI the more attenuated the mutant strain. Similar experiments were performed with a pure inocula of wild-type or Δ*livHMGF* strain bacteria to calculate the bacterial CFU for each strain at specific time points or to monitor the progress of infection identifying mice likely to progress to fatal disease according to previously established criteria (6).

Statistical analysis. All of the in vitro growth curves were performed in triplicates and are represented as means and standard deviations. The results of growth curves, radioactive uptake, and binding assays were analyzed by using two-tailed Student *t* tests. The results of survival experiments were compared by using the log-rank method, and target organ CFU levels were analyzed by using the Mann-Whitney U test. The statistical validity of the results for CIs is represented by 95% confidence intervals.

RESULTS

Selection of ABC transporters for investigation. The ABC transporters chosen for these studies were identified from the annotated genome of the capsular serotype 4 strain of *S. pneumoniae* (TIGR4) (40). This genome contains 24 loci consisting of three or more adjacent genes that encode putative components of ABC transporters and are likely to be transcribed as an operon, 10 of which have been previously investigated. Eleven of the previously undescribed ABC transporters

TABLE 2. Primers used in this study

Primer	Sequence[a]
Sp0090.1	GCTCTAGACATTGAGAGAGACAACTGG
Sp0090.2	CGCTCTAGACAAGAAGTAAGGGAAC
Sp0149.1	GCTCTAGAGGCTCTTGCAGCTTGCGG
Sp0149.2	CGCTCTAGAGGCTTTCGTTTGTAGCGTC
Sp0610.1	GCTCTAGATTACGGAGACTACCACG
Sp0610.2	CGCTCTAGATCCACCAGATAGCATGCC
Sp0710.1	GCTCTAGATTGGGCGTTACGATTG
Sp0710.2	CGCTCTAGAGTGCCTGTCCACTTTC
Sp0750.1	GCTCTAGACGCGCTGTTAGCCCTAGG
Sp0750.2	GCTCTAGATGATACTGCACGCATGGC
Sp846.1	GCTCTAGATGTTAGCAGGCCTTCTTG
Sp846.2	CGCTCTAGAGCCCCTGCAATTTCAAC
Sp1690.1	GCTCTAGATTGCGCTAGCGGCTGTTG
Sp1690.2	GCTCTAGAGCTTCCTCCACCACTACG
Sp1798.1	GCTCTAGATGACTGTCCCCGGTTTAG
Sp1798.2	CGCTCTAGATTGTTGATTGGTCCTCCC
Sp1826.1	GCTCTAGACGACTGCTTCTTCATCTG
Sp1826.2	CGCTCTAGAATTGCCCGTCCTGTACC
Sp2084.1	GCTCTAGATTTGGGCTTGTTGCCTG
Sp2084.2	CGCTCTAGATGTGACCACTTGTTGACC
Sp2108.1	GCTCTAGAACTGCTACACTTGCTAG
Sp2108.2	CGCTCTAGAACCAAGGCTACCTAC
Cm.1	TTATAAAAGCCAGTCATTAG
Cm.2	TTTGATTTTTAATGGATAATG
Sp1	TCGAGATCTATCGATGCA
Sp3	GGATCCATATGACGTCGA
EryF	ATGAACAAAAATATAAAATA
EryR	TTATTTCCTCCCGTTAAATAAT
Sp0749F	CACTGACAATGCCAGTGACTATGC
Ery-Sp0749R	TATTTTATATTTTTGTTCATAAGATTCACTC TTTCTATTTATAA
Ery-Sp0753F	ATTATTTAACGGGAGGAAATAAAACATTC CAGTGGATTGTTTTAG
Sp0754R	GCGGAATATTGACTGTATGGGAG
Sp0749Fwd	CGGGATCCTGTGGAGAAGTGAAGTCTGGA
Sp0749Rv	CGGGATCCTTATGGTTTTACAACTTCTGC
Sp0749RT1	CGATGCAGACCACAACAC
Sp750RT2	CCTAGGGCTAACAGCGC
750RT1.1	GATGGGGGTTACTCCAGG
751RT2.1	CCCAGAGTTGCTACCGC
751RT1.1	GGTGCGATTGTTTCGG
752RT2.1	GGTTCCGTAGCAAGGG
Sp0752RT1	GGCCGTTTAATCGCTCAAG
Sp0753RT2	GGGCGCGTCCCATGGCAAG
753RT1.1	GGAGAATCGTCCTATCAG
754RT2.1	CAGGCAGACGGTGCAAACC

[a] Restriction enzyme sites in 5′ linkers are underlined.

present in the TIGR4 genome of *S. pneumoniae* were selected for further investigation (Sp0090-3, Sp0148-52, Sp0607-10, Sp0708-11, Sp0749-53, Sp0846-8, Sp1688-90, Sp1796-8, Sp1824-6, Sp2084-7, and Sp2108-10). The results of BLAST searches using the derived amino acid sequence for each gene within the putative operons are shown in Table 3. Most of these ABC transporter proteins have >90% identity and similarity at the amino acid level to proteins encoded by genes in the *S. pneumoniae* R6 strain, an avirulent laboratory strain of *S. pneumoniae* derived from a capsular serotype 2 strain. However, there are no homologues of the Sp1796-8 operon in the R6 genome, and BLAST searches versus the 18 other available *S. pneumoniae* genomes demonstrated that the Sp1796-8 operon was also absent from the genomes of a further five strains (CDC1087-00, D39, G54, Hungary 19A-6, and Sp23-BS72). In contrast, the SBPs for almost all of the remaining ABC transporters were highly conserved between *S. pneumoniae* strains, with 97% or greater levels of identity between the deduced amino acid sequence of the TIGR4 SBP with the sequence of the equivalent SBP for all 19 *S. pneumoniae* strains with available genome data. The exception was Sp1826, which had between 93 and 95% identity to proteins encoded by genes present in 18 strains and 99% identity to the remaining strain (CDC0288-44). As expected, the majority of the predicted proteins have at least 50% identity and 60% similarity to proteins encoded by other streptococci (Table 3). In contrast, the genes in the putative Sp1688-90, Sp1824-26, and Sp2084-87 operons have no close homologues in streptococci. Sp1688-90 has >70% identity and >85% similarity to the amino acid sequence of predicted proteins encoded by Pm1760-62 of the gram-negative bacteria *Pasteurella multocida*, and Sp1824-26 and Sp2084-87 both have lower levels of identity and similarity to predicted proteins from a variety of unrelated bacteria. The mean G+C contents of Sp1688-90 and Sp1824-6 are 37 and 33.5%, respectively, which is significantly lower than the G+C content of the complete genome of TIGR4 (39.7%) (6, 40). The mean G+C content of Sp2084-7 was 39.0%, which is not significantly different from that of the complete genome of TIGR4. These data suggest that Sp1688-90, Sp1824-6, and possibly Sp2084-7 are contained within regions of the *S. pneumoniae* genome that could have been acquired by horizontal transfer from an unrelated species.

Construction of ABC transporter mutant strains. To investigate the role of the selected ABC transporters during in vivo growth and virulence, mutant strains of the 0100993 strain of the capsular serotype 3 *S. pneumoniae* were constructed by using IDM. Mutations were designed to disrupt one gene, usually the first, within the putative operon of each ABC transporter (Fig. 1) and were successfully obtained for 10 of the 11 chosen ABC transporters. Although the disruption construct was made successfully, no mutants were obtained by disrupting Sp0710 despite repeated transformations. The stability of each mutation was assessed by culturing the mutant strains in THY broth in the absence of antibiotic (and therefore selective pressure) for two 8-h growth cycles, followed by plating onto plain and antibiotic plates. The Sp2084$^-$ mutation was unstable (19 chloramphenicol-resistant colonies recovered compared to >1,000 on plain plates), preventing further investigation of this mutant, whereas the mutations in the remaining mutant strains were stable with similar numbers of colonies on the plain and antibiotic plates. All of the mutant strains had similar growth in THY compared to the wild-type strain when measured by monitoring the OD_{580} over time (data not shown).

Phenotypes of mutant strains containing disruptions in genes encoding ABC transporters. The phenotypes of the mutant strains were investigated using mixed inocula and CIs to identify subtle defects affecting in vitro growth and to assess their virulence compared to the wild-type strain in animal models of infection. CIs were obtained for growth in complete medium (THY) and a physiologically relevant fluid (human blood) and in mouse models of pulmonary and systemic infection using bacteria recovered from the lungs and spleen, respectively, to calculate the CI (Table 4). We were unable to establish a nasopharyngeal colonization model for the serotype 3 strain in which the mutants were constructed, preventing assessment of the role of these ABC transporters during col-

TABLE 3. BLAST alignments of the derived amino acid sequences of the investigated *S.pneumoniae* ABC transporters

Locus tag[a]	Size (no. of amino acids)	Protein	Organism	% Identity/similarity[b]	Possible substrate (transport classification)[c]
Sp0090	319	EfaeDRAFT_2526	*Enterococcus faecium*	61/80 (311)	Sugar (CUT1 3.A.1.1)
Sp0091	307	EfaeDRAFT_2527	*Enterococcus faecium*	60/79 (300)	Sugar (CUT1 3.A.1.1)
Sp0092	491	EfaeDRAFT_2538	*Enterococcus faecium*	53/70 (486)	Sugar (CUT1 3.A.1.1)
Sp0148	276	SMU.1942c	*Streptococcus mutans*	55/76 (237)	Methionine (MUT 3.A.1.24)
Sp0149	284	SPs1626	*Streptococcus pyogenes*	67/81 (278)	Methionine (MUT 3.A.1.24)
Sp0150	457	SMU.1940c	*Streptococcus mutans*	70/85 (457)	Methionine (MUT 3.A.1.24)
Sp0151	353	SsiuDRAFT_0064	*Streptococcus suis*	80/90 (352)	Methionine (MUT 3.A.1.24)
Sp0152	230	SPs1624	*Streptococcus pyogenes*	71/89 (230)	Methionine (MUT 3.A.1.24)
Sp0607	219	SMU.1522	*Streptococcus mutans*	74/85 (213)	Amino acid (PAAT 3.A.1.3)
Sp0608	219	SMU.1521	*Streptococcus mutans*	62/81 (222)	Amino acid (PAAT 3.A.1.3)
Sp0609	254	StheL01000593	*Streptococcus thermophilus*	60/73 (232)	Amino acid (PAAT 3.A.1.3)
Sp0610	252	StheL01000592	*Streptococcus thermophilus*	82/90 (251)	Amino acid (PAAT 3.A.1.3)
Sp0708	215	SsuiDRAFT_0032	*Streptococcus suis*	60/77 (209)	Amino acid (PAAT 3.A.1.3)
Sp0709	252	SGO_0983	*Streptococcus gordonii*	88/94 (252)	Amino acid (PAAT 3.A.1.3)
Sp0710	225	SGO_0984	*Streptococcus gordonii*	89/95 (225)	Amino acid (PAAT 3.A.1.3)
Sp0711	206	SGO_0985	*Streptococcus gordonii*	87/94 (226)	Amino acid (PAAT 3.A.1.3)
Sp0749	386	SAG1582	*Streptococcus agalactiae*	53/73 (390)	BCAA (HAAT 3.A.1.4)
Sp0750	289	SAK_1597	*Streptococcus agalactiae*	83/93 (289)	BCAA (HAAT 3.A.1.4)
Sp0751	318	gbs1630	*Streptococcus agalactiae*	73/88 (252)	BCAA (HAAT 3.A.1.4)
Sp0752	254	SsuiDRAFT_0078	*Streptococcus suis*	85/93 (254)	BCAA (HAAT 3.A.1.4)
Sp0753	236	SsuiDRAFT_0077	*Streptococcus suis*	87/96 (236)	BCAA (HAAT 3.A.1.4)
Sp0846	511	Spy1227	*Streptococcus pyogenes*	81/91 (508)	Ribonucleoside (CUT2 3.A.1.2)
Sp0847	352	SAK_1051	*Streptococcus agalactiae*	77/88 (353)	Ribonucleoside (CUT2 3.A.1.2)
Sp0848	318	Spy0928	*Streptococcus pyogenes*	80/92 (318)	Ribonucleoside (CUT2 3.A.1.2)
Sp1688	277	PM1760	*Pasteurella multocida*	80/91 (277)	Sugar (CUT1 3.A.1.1)
Sp1689	294	PM1761	*Pasteurella multocida*	79/93 (291)	Sugar (CUT1 3.A.1.1)
Sp1690	445	PM1762	*Pasteurella multocida*	70/85 (407)	Sugar (CUT1 3.A.1.1)
Sp1796	538	SsuiDRAFT_0524	*Streptococcus suis*	78/89 (537)	Sugar (CUT1 3.A.1.1)
Sp1797	305	SsuiDRAFT_0525	*Streptococcus suis*	82/94 (294)	Sugar (CUT1 3.A.1.1)
Sp1798	305	SsuiDRAFT_0526	*Streptococcus suis*	84/95 (303)	Sugar (CUT1 3.A.1.1)
Sp1824	563	Lxx14070	*Leifsonia xyli*	31/48 (539)	Cation 2 (BIT 3.A.1.0)
Sp1825	336	STH2752	*Symbiobacterium thermophilum*	45/59 (343)	Cation 2 (BIT 3.A.1.0)
Sp1826	355	Lxx14040	*Leifsonia xyli*	30/48 (323)	Cation 2 (BIT 3.A.1.0)
Sp2084	291	RUMOBE_00498	*Ruminococcus obeum*	46/65 (287)	Phosphate (PhoT 3.A.1.7)
Sp2085	287	Cthe_1604	*Clostridia thermocellum*	61/98 (284)	Phosphate (PhoT 3.A.1.7)
Sp2086	271	DORLON_00312	*Dorea longicatena*	59/80 (287)	Phosphate (PhoT 3.A.1.7)
Sp2087	250	BACCAP_00261	*Bacteroides capillosus*	72/86 (253)	Phosphate (PhoT 3.A.1.7)
Sp2108	423	M_28Spy1048	*Streptococcus pyogenes*	53/67 (420)	Maltodextrin (CUT1 3.A.1.1)
Sp2109	435	Spy1301	*Streptococcus pyogenes*	66/82 (430)	Maltodextrin (CUT1 3.A.1.1)
Sp2110	280	SsuiDRAFT_0440	*Streptococcus suis*	85/93 (280)	Maltodextrin (CUT1 3.A.1.1)

[a] That is, the TIGR4 genome locus tag number.
[b] The length, in amino acids, compared is indicated in parentheses.
[c] Transport classification and subfamily and number according to the transport classification system based on sequence similarity (32, 45; see also http://www.tcdb.org/).

onization. Most of the mutants had CIs close to 1.0 in THY, suggesting that disruption of the target ABC transporters had no effect on growth in complete medium. The exception was ΔSp0750 which had mildly impaired growth in THY. In contrast, in normal physiological fluid such as human blood, the mutants ΔSp0090, ΔSp0149, ΔSp0750, ΔSp1824, and (to a lesser extent) ΔSp0610 had reduced CIs, suggesting that these mutants have a particular problem growing in physiological fluid compared to THY. The in vivo CIs for most mutant strains mirrored the CIs for growth in blood, with these strains showing no impairment in CI in blood also being fully virulent (ΔSp0846, ΔSp1690, and ΔSp1796), whereas strains with impaired CIs in blood also had a reduced CI during infection. These data indicate that for these mutant strains, impaired growth under physiological conditions is associated with a reduced ability to cause invasive infection. In addition, Δ*S*p2108 had some impairment in virulence after i.p. inoculation without any impairment in CI in growth in blood. For both systemic and pulmonary infections, the ΔSp0750 and ΔSp0149 strains were markedly more attenuated in virulence than the other mutant strains with a CI reduced out of proportion to the reduced CI in blood.

Construction of a deletion mutant of Sp0750-53. The data obtained with the disruption mutant strains suggested that the ABC transporters encoded by Sp0149-52 and Sp0749-53 have the most crucial roles during *S. pneumoniae* infection (Table 4). BLAST alignments of the predicted proteins encoded by Sp0149-52 suggested these genes encode a methionine ABC transporter protein, but the function of this ABC transporter was not investigated further at this stage (37). BLAST alignments of Sp0749-53 showed the proteins encoded by these genes have high levels of similarity to predicted BCAA ABC transporter proteins (Table 3), clearly indicating the likely function of this ABC transporter. Auxotrophy for BCAA due to mutations affecting BCAA synthesis has been shown to affect the virulence of *Mycobacterium bovis* and *Burkholderia*

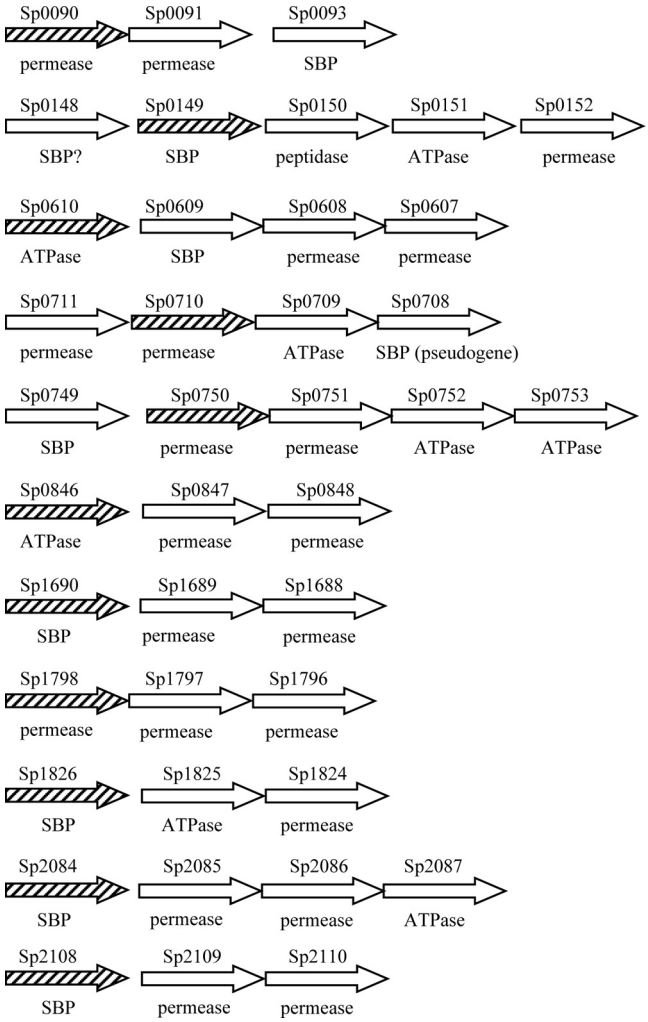

FIG. 1. Schematic diagram showing the organization of loci encoding ABC transporter components investigated in the present study. Each arrow represents a single gene, which are not drawn to scale. Gaps between genes correspond to gaps of greater than 50 bp between the stop codon of the upstream gene and the ATG of the downstream gene. The TIGR4 gene number is given above each gene, and whether the gene encodes a putative SBP, ATPase or membrane protein (permease) is written beneath. The diagonally shaded gene in each locus (▨) was targeted by IDM using pID701 containing an internal portion of the target gene.

pseudomallei (3, 24) but, as far as we are aware, no role has yet been described for BCAA transporters in bacterial virulence. We therefore elected to investigate the Sp0749-53 transporter in more detail and have named the genes *livJHMGF* to correspond to their *E. coli* orthologues (1).

To confirm the genetic organization of Sp0749-0753, RT-PCR was performed using RNA from strain 0100993 as the template and primers designed to amplify a product that spans the junctions of the genes present in the putative operon (Table 2). The lack of DNA contamination was confirmed by the absence of products for reactions with no added RT enzyme (data not shown), and RT-PCR products were sequenced to confirm their identities. The *livJHMGF*/Sp0749-53 region is 4,831 bp in length and consists of five genes, *livJ*/Sp0749 (encoding an SBP lipoprotein), *livH*/Sp0750 (encoding a membrane protein permease), *livM*/Sp0751 (encoding a membrane protein permease), *livG*/Sp0752 (encoding an ATPase) and *livF*/Sp0753 (encoding an ATPase) (Table 3 and Fig. 2A). The RT-PCR results matched with the operon structure deduced from the TIGR4 genome sequence, suggesting that *livJ* is not cotranscribed with *livH* (corresponding to the relatively large intergenic region between these two genes of 268 bp) and that *livH*, *livM*, *livG*, and *livF* are all cotranscribed (Fig. 2A and B). Although the RT-PCR product obtained using primers spanning the junction of *livM* and *livG* was smaller than the expected size, sequencing confirmed it represented a product of the 5′ region of *livM* and 3′ region of *livG* and conformed exactly in length and almost exactly in nucleotide sequence to that predicted from the genome data for the TIGR4 strain. RT-PCR between *livF* and Sp0754 did generate a cDNA fragment, but sequencing demonstrated this was a nonspecific product. For further investigation of the function of *livJHMGF*, a mutant strain in the *S. pneumoniae* 0100993 background was constructed in which *livHMGF* were deleted using a construct made by overlap extension PCR (Fig. 2C and D). Correct deletion of these genes in the Δ*livHMGF* mutant strain was confirmed by PCR and sequencing.

In vivo phenotypes of the Δ*livHMGF* mutant strain. To investigate the effect of deletion of *livHMGF* on the virulence of *S. pneumoniae*, CIs were determined in the pulmonary and systemic mouse models of infection. The CIs for the Δ*livHMGF* deletion mutant were reduced in models of pneumonia and septicemia showing a significant attenuation in virulence in these models of infection (Table 5), although the results were

TABLE 4. In vitro and in vivo phenotype analysis of *S. pneumoniae* ABC transporter mutant strains using CI values[a]

Strain	CI (95% confidence interval) with various sample types			
	THY	Blood	i.p.	i.n.
ΔSp0090	1.10 (0.24–2.0)	0.39 (0.31–0.47)	0.49 (0.37–0.61)	0.43 (0.23–0.63)
ΔSp0149	0.95 (0.67–1.22)	0.46 (0.37–0.57)	0.067 (0.031–0.10)	0.041 (0.021–0.10)
ΔSp0610	1.40 (1.02–1.77)	0.71 (0.58–0.83)	0.70 (0.53–0.86)	0.64 (0.15–1.20)
ΔSp0750	1.06 (0.86–1.25)	0.40 (0.20–0.60)	0.17 (0.078–0.26)	0.018 (0.002–0.032)
ΔSp0846	0.95 (0.67–1.22)	1.25 (0.90–1.60)	0.61 (0.22–1.00)	0.54 (0.30–0.79)
ΔSp1690	0.97 (0.77–1.18)	0.92 (0.56–1.27)	0.89 (0.41–1.38)	ND[b]
ΔSp1796	0.82 (0.59–1.04)	0.78 (0.56–0.99)	1.30 (0.93–1.71)	ND
ΔSp1824	0.95 (0.25–1.65)	0.66 (0.43–0.89)	0.59 (0.34–0.84)	0.33 (0.01–0.67)
ΔSp2108	1.05 (0.71–1.40)	1.25 (0.92–1.57)	0.39 (0.29–0.49)	1.00 (0.36–1.64)

[a] For mouse experiments, $n = 3$ to 10.
[b] ND, not done.

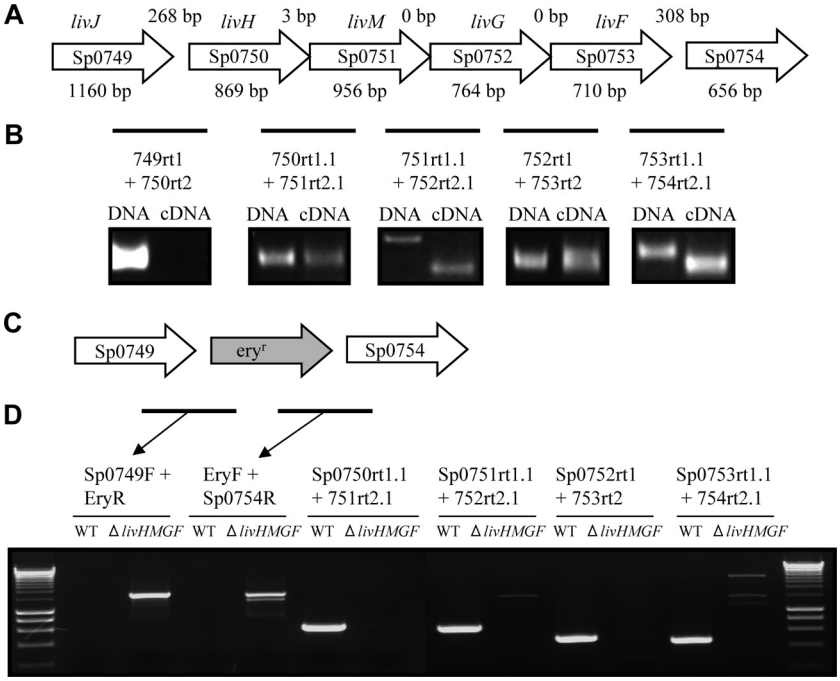

FIG. 2. (A) Structure of the *livJHMGF* locus. Each arrows represents one gene and contains the Sp number within the arrow and the gene annotation above the arrow. The size in base pairs of each gene is given below the arrow, and the number of base pairs between the stop codon of the upstream gene and the ATG of the downstream gene is given above the arrows in the corresponding gap. (B) Results of RT-PCR assessment of the transcriptional linkage between the *livJHMGF* genes. The bar represents the target product relative to the genes shown in panel A for each primer pair (named beneath the bar). A picture of the ethidium bromide-stained agarose gels showing the PCR products obtained using these primers and either DNA or cDNA as the template is given with each primer pair. (C) Representation of the structure of the *livJHMGF* locus in the Δ*livHMGF* strain. (D) Ethidium bromide-stained agarose gels showing the PCR products obtained from the Δ*livHMGF* and wild-type strain, confirming replacement of the *livHMGF* with the erythromycin resistance cassette (ery^r in the figure) in the Δ*livHMGF* strain. Bars represent expected products for the primer pairs given above the lanes for their corresponding PCR products.

higher than those obtained with the ΔSp0750 IDM mutant strain (Table 4). CIs were also obtained for a Δ*livHMGF* strain in the TIGR4 background in models of nasopharyngeal colonization, systemic infection, and pneumonia (Table 5). The TIGR4 Δ*livHMGF* strain was significantly attenuated in virulence in the pneumonia model but not in the nasopharyngeal colonization model and, in contrast to the serotype 3 0100993 Δ*livHMGF* strain, had only a statistically not significant (the confidence intervals overlapped with 1.0) small decrease in the CI for the systemic model of infection. To assess the ability of the Δ*livHMGF S. pneumoniae* strain to cause fatal disease, groups of 10 CD1 mice strain were inoculated i.n. with 10^7 CFU of the wild-type *S. pneumoniae* 0100993 strain or the Δ*livHMGF* deletion strain, and the progress of infection was monitored (6) (Fig. 3A). There was no difference in the survival of mice inoculated with the wild-type and Δ*livHMGF* strains, demonstrating that despite the impaired virulence of the Δ*livHMGF* strain when in competition with the wild-type strain this strain is still able to cause fatal disease. In addition, bacterial CFU in mouse lungs culled 48 h after inoculation of either the wild-type or Δ*livHMGF* strains were not significantly different (Fig. 3B). These data show that the loss of LivJHMGF only impairs virulence significantly in a competitive model of infection.

TABLE 5. In vivo phenotype analysis of the Δ*livHMGF S. pneumoniae* strains compared to the wild-type strain using CI values for three different serotype backgrounds[a]

Capsular serotype	CI (95% confidence interval) obtained by various routes		
	n.p. (nasal wash)	i.n. (lung)	i.p. (spleen)
3	ND[b]	0.11 (0.004–0.58)	0.076 (0.040–0.094)
4	1.05 (0.68–1.41)	0.018 (0.001–0.34)	0.62 (0.16–1.08)

[a] For these experiments, $n = 5$ to 10. n.p., nasopharyngeal.
[b] ND, not done (there was no nasopharyngeal model for this strain).

In vitro phenotypes of the Δ*livHMGF* strain. When compared using the OD_{580}, the growth of the Δ*livHMGF* mutant strain was similar to the growth of the wild-type strain in THY and in a defined chemical medium CDEM with or without supplementation with the BCAAs valine, isoleucine, or leucine (data not shown). As shown for the ΔSp0750 IDM mutant strain (Table 4), the CI of the Δ*livHMGF* mutant strain compared to the wild-type strains in vitro after growth in blood was impaired (CI = 0.56, confidence intervals of 0.34 to 0.78), but this was not corrected by the addition of 10 mg of valine, isoleucine, or leucine/ml impaired (CI = 0.44, confidence intervals of 0.15 to 0.74). Uptake of azaleucine by BCAA ABC transporters causes toxicity and impaired growth of gram-negative bacteria (14, 18), which is reduced if BCAA uptake is inactivated. However, azaleucine in concentrations of up to 100 μg ml^{-1} was not toxic to *S. pneumoniae* (data not shown), and azaleucine toxicity could not therefore be used to assess

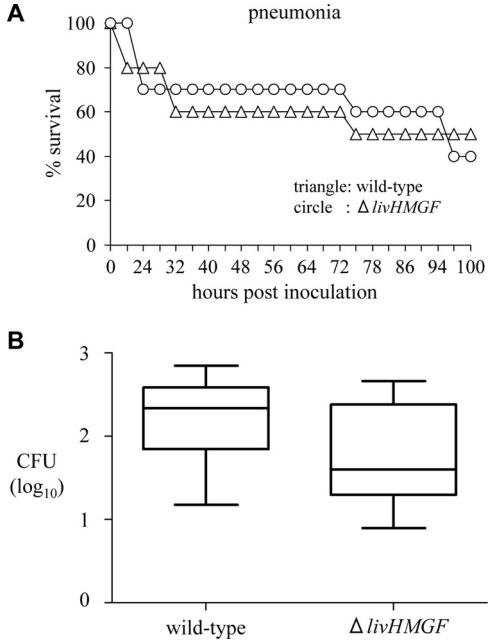

FIG. 3. Assessment of the virulence of the Δ*livHMGF* strain in mouse models of infection. (A) Time course for the development of fatal infection for groups of 10 mice given 5×10^6 CFU of either the wild-type or the Δ*livHMGF* strain ($P = 0.88$, log-rank test). (B) Log_{10} ml^{-1} bacterial CFU recovered from the lungs of mice 24 h after inoculation with 5×10^6 CFU of either the wild-type or Δ*livHMGF* strain. The results are shown as box-and-whisker diagrams of the median $\log_{10}$ ml^{-1} bacterial CFU and the interquartile range for data obtained from two identical experiments, each with five mice per strain.

BCAA uptake. Furthermore, attempts to confirm that *livJHMGF* does encode an BCAA ABC transporter by comparison of leucine and isoleucine uptake by the wild-type and Δ*livHMGF* strains were unsuccessful. Although significant uptake of the positive control substrate maltose was demonstrated for both the wild-type and the Δ*livHMGF* strains, no significant uptake of [^{14}C]leucine (Table 6) or [^{14}C]isoleucine (data not shown) could be detected under these experimental conditions even by the wild-type strain, precluding the use of uptake assays to assess the function of LivJHMGF.

LivJ is a BCAA binding protein. Since the SBP component provides substrate specificity for ABC transporters, to identify the substrate(s) of the LivJHMGF ABC transporter the binding of labeled amino acids to the LivJ (Sp0749) SBP was assessed. An N-terminally histidine-tagged LivJ (His_6-LivJ) was expressed in *E. coli* and purified to ca. 95% purity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that most of the purified protein was found in a single band of ~40 kDa, which is compatible with the expected mass for His_6-LivJ. There was an additional band with a mass of ~80 kDa. Peptide mass fingerprinting confirmed that the 40-kDa band was His_6-LivJ and that the 80-kDa band consisted of a dimer of His_6-LivJ. The ligand-binding properties of His_6-LivJ were investigated by measuring changes in intrinsic protein fluorescence, using tyrosine fluorescence (excitation at 280 nm) since the protein lacks any tryptophan residues. We were able to observe small but reproducible protein-dependent fluorescence changes in His_6-LivJ upon the addition of 0.32 μM isoleucine, 3.2 μM leucine, and 16 μM valine (Fig. 4). There was no further fluorescence change with the addition of excess ligand, suggesting binding was saturated (Fig. 4). There were also no changes in fluorescence with the addition of 50 μM concentrations of the non-BCAAs proline, glycine, and alanine (data not shown). To estimate a relative affinity of the protein toward these three BCAAs, the lowest concentration of ligand for which we could detect a fluorescence change over the slow decrease in signal due to photobleaching was determined. Using 1.6 μM concentrations of ligand we could only detect isoleucine and leucine binding, and using 0.32 μM concentrations of ligand we could only detect isoleucine binding (Fig. 4 and data not shown). These data suggest that LivJ (Sp0749) is able to bind BCAA with a preference for isoleucine over leucine over valine and that the protein is likely to bind isoleucine in the submicromolar range that is typical for the physiological substrate of other ABC transporters.

The ligand-binding properties of His_6-LivJ were also investigated by using a radioactive binding assay. The His_6-LivJ lipoprotein bound to [^{14}C]isoleucine and to a lesser extent [^{14}C]leucine but not to the negative control ligand [α-^{14}C]aminoisobutyric acid (AIB) (Fig. 5A). Competitive binding experiments using putative ligands to inhibit [^{14}C]isoleucine binding to His_6-LivJ protein demonstrated that leucine and, to a lesser extent, valine and threonine inhibited [^{14}C]isoleucine binding (Fig. 5B). Overall, the results of the fluorescence and radioactive binding studies show that the substrates of the *S. pneumoniae* LivJHMGF ABC transporter are BCAAs, with high-affinity binding of LivJ to isoleucine, moderate affinity binding to leucine and the least affinity toward valine.

DISCUSSION

Genes encoding components of ABC transporters make up a significant portion of many bacterial genomes, including *S. pneumoniae* (12, 40). Through controlling uptake or export of a wide range of substrates, ABC transporters have a central role in modulating the bacterial interactions with the environment, including the host for pathogens. Hence, it is perhaps not surprising that STM screens for virulence determinants frequently have identified ABC transporters (9, 10, 15, 22, 26) and that detailed characterization of individual *S. pneumoniae* ABC transporters have shown that several affect the development of infection through a variety of mechanisms (6, 17, 27, 34, 44). We have screened 11 *S. pneumoniae* ABC transporters that have not, as far as we are aware, previously been investigated for their roles in virulence. Mixed infections and CIs are

TABLE 6. [^{14}C]leucine and [^{14}C]maltose uptake by wild-type and Δ*livHMGF* strains

Strain	Substrate	Uptake (mmol/mg of protein) at:		
		60 s	120 s	180 s
Wild type	[^{14}C]leucine	0.44	0.47	0.35
	[^{14}C]maltose	12.8	30.4	40.4
Δ*livHMGF*	[^{14}C]leucine	0.41	0.62	0.71
	[^{14}C]maltose	13.0	32.7	44.6

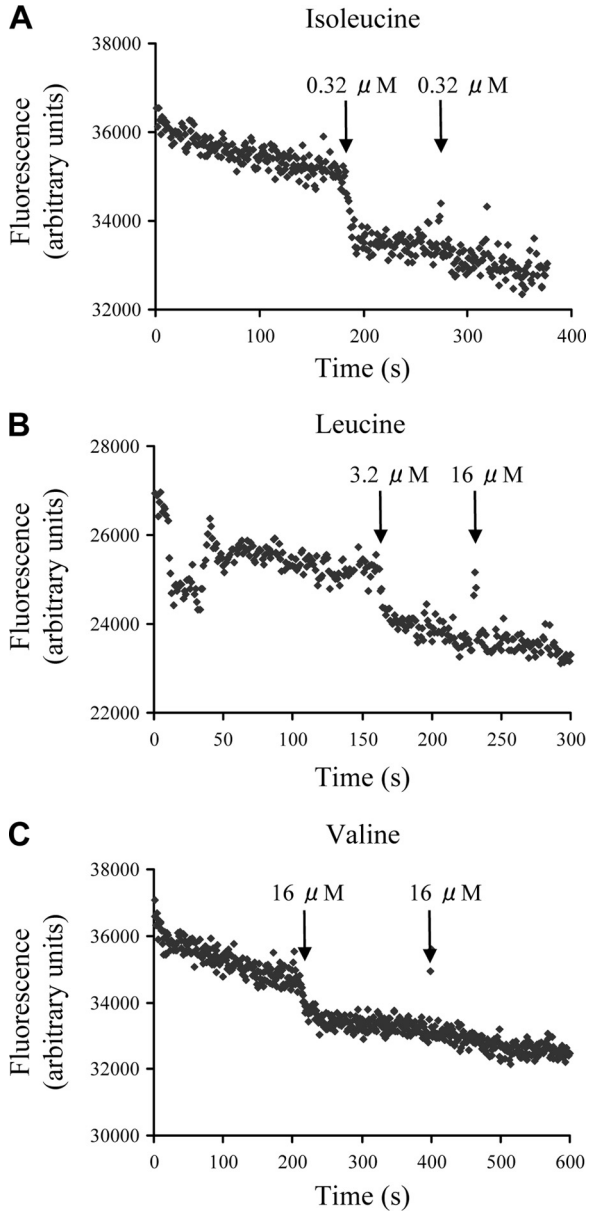

FIG. 4. Tryptophan fluorescence spectroscopy of purified His_6-LivJ after addition of 0.32 μM isoleucine (A), 3.2 μM leucine (B), or 16 μM valine (C) (marked by the first arrow). The addition of excess ligand (marked by the second arrow) had no further effect on fluorescence, nor did the addition of 50 μM proline, glycine, or alanine (data not shown). Fluorescence was measured by using arbitary units and an excitation wavelength of 280 nm with an emission wavelength of 309 nm.

highly sensitive at identifying virulence defects and only require relatively small numbers of animals and so were used to assess the relative virulence of mutant strains containing disrupted ABC transporter genes to the wild-type strains (5, 6). We were unable to obtain any mutants affecting the putative amino acid transporter encoded by Sp0707-0711, perhaps since this transporter is important for *S. pneumoniae* viability, and the insertional duplication mutant affecting the putative phosphate transporter encoded by Sp2084-2087 was too unstable for further investigation. Of the nine ABC transporter mutant strains successfully constructed, six were impaired in full virulence in mouse models of sepsis and/or pneumonia (ΔSp0090, ΔSp0149, ΔSp0610, ΔSp0750, ΔSp1824, and ΔSp2108). Their putative roles included sugar, cation, BCAA, and other amino acid transport, and for the majority the CIs in blood were similar to the CIs in the infection model, suggesting that their effects on virulence are likely to be due to their role during growth under physiological conditions. These results indicate that ABC transporters frequently influence *S. pneumoniae* disease pathogenesis, demonstrating why mutations affecting lipoprotein and therefore SBP processing by *S. pneumoniae* affect virulence (21, 29). The majority of the loci encoding ABC transporters investigated in the present study have equivalents in other streptococci, and it is likely that some of these will also be required for full virulence.

For four of the ABC transporter mutant strains the impairment in virulence was relatively small, comparable to the effect of the loss of a single iron transporter (17), and this may be because their functions are partially redundant. For example, BLAST alignments suggest that several of the ABC transporters we have investigated encode sugar transporters, and disrupting the function of one could be compensated for by the others or even by non-ABC transporter uptake mechanisms such as phosphoenolpyruvate-dependent sugar transporters (40). In addition, in the host there are a variety of sugar substrates available, and the inability of *S. pneumoniae* to use a single sugar due to impaired uptake may not be critical due to the availability of other sugars. Dual mutations in genes encoding components of ABC transporters with related functions may have a much more marked effect on virulence as has been shown for the Piu and Pia iron transporters (6). However, too few functional data on the ABC transporters were investigated here to be able to determine which ABC transporters should be selected for dual mutation. The effects of disruption of these ABC transporters on virulence indicates bacterial functions that are likely to be important during infection, which our data suggest includes sugar, amino acid, and cation uptake. Although cation uptake is a well-recognized requirement for the virulence of *S. pneumoniae* and other bacteria (6, 17, 20, 25), sugar and amino acid transport is less well recognized. Identification of the specific substrate for each transporter will help define the precise physiological requirements for *S. pneumoniae* virulence but will require painstaking screening of a range of potential substrates using in vitro phenotypes and binding and uptake assays. In addition to ABC transporters encoded for by groups of genes investigated in this and previous studies, there are many additional ABC transporter components encoded by isolated single genes or gene pairs within the TIGR4 genome, some of which STM screens suggest may affect virulence (15, 22). These ABC transporter components also warrant further investigation, although their specific putative functions will in general be even less apparent than those encoded by several adjacent genes in putative operons.

Two mutant strains had a marked effect on virulence when analyzed using CIs. One contained a disrupted copy of Sp0149, part of a potential methionine transporter which is the subject of continued investigation in our laboratory. The second contained a disrupted copy of Sp0750, part of an operon whose

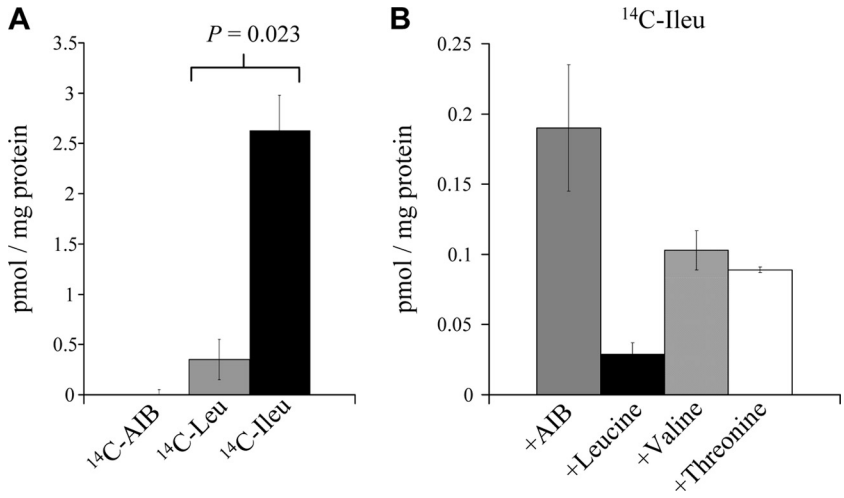

FIG. 5. Radioactive binding assays to purified His_6-LivJ. (A) Degree of binding of ^{14}C-AIB (negative control) and the BCAAs [^{14}C]leucine and [^{14}C]isoleucine to His_6-LivJ expressed as pmol of substrate per mg of His_6-LivJ. No significant binding to AIB was detected. For the differences between [^{14}C]leucine and [^{14}C]isoleucine, $P = 0.023$ (Student t test). (B) Competitive blocking of [^{14}C]isoleucine binding to His_6-LivJ by the addition of excess (500 μM) AIB, leucine, valine, or threonine. Compared to AIB, $P = 0.005$ for leucine, 0.014 for valine, and <0.001 for threonine. Compared to leucine, $P < 0.05$ for both valine and threonine (one-way analysis of variance).

function is strongly indicated by BLAST searches to be BCAA uptake. The genes at the Sp0749-53 loci are annotated as *livJHMGF* in the TIGR4 genome (40) and are organized in a fashion similar to that of the equivalent BCAA of *E. coli*, with the SBP component encoded by *livJ* that is transcribed as a separate transcript to the remaining genes within the operon *livHMGF* (19). Tryptophan fluorescence spectroscopy and radioactive binding assays using [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine demonstrated that LivJ binds specifically to BCAA, strongly supporting that *livJHMGF* encodes a BCAA ABC transporter. Both the tryptophan fluorescence spectroscopy and the radioactive binding assays suggest that LivJ has the highest affinity for isoleucine and then leucine, with the least affinity for valine.

Growth of the Δ*livHMGF* strain was not affected in media depleted in BCAA, the toxic BCAA analogue azaleucine did not impair *S. pneumoniae* growth, and we were unable to identify significant uptake of the BCAA leucine by *S. pneumoniae*. Hence, in the conditions used for these experiments (i.e., the complete medium THY and the defined medium CDEM) there seems to be very little BCAA uptake by *S. pneumoniae*. In contrast, in mouse models of infection the Δ*livHMGF* and the IDM mutant strain with a disrupted copy of *livH* were both significantly outcompeted by the wild-type strain. These data suggest that there is an important role for the *S. pneumoniae* BCAA specifically during in vivo growth, a conclusion that is supported by previous publications which have shown that disruption of BCAA synthesis affects the virulence of the unrelated pathogens *B. pseudomallei* and *M. bovis* (3, 24). However, the effect of loss of LivHMGF on virulence was only detectable in competitive infection experiments, and after i.n. inoculation with either the Δ*livHMGF* or wild-type strain, bacterial CFU in target organs and progression of infection were similar. When transferred to the TIGR4 strain, the Δ*livHMGF* deletion affected virulence in the pneumonia model but not in the model of systemic infection, a surprising result given that the effect on virulence was most marked in the systemic model for the serotype 3 strain. These data have no immediately apparent explanation and provide further evidence that the effect of mutations of *S. pneumoniae* genes depends on strain background. Overall, the data suggest that LivJHMGF does not have a powerful effect on *S. pneumoniae* disease pathogenesis, perhaps because the *S. pneumoniae* genome contains genes encoding enzymes required for BCAA synthesis (Sp0445-50) (24, 40). These may partially compensate for impaired BCAA uptake in vivo, perhaps to a variable degree between strains, so the effects of the Δ*livHMGF* mutation on in vivo growth are only detectable if competing against a wild-type strain and depend on strain background. Exactly why the loss of BCAA affects virulence is not clear. The most obvious explanation is a nutritional requirement for BCAA in vivo, and the differences in CI between sites could be explained by variations in bacterial demand for BCAA, perhaps related to differences in bacterial replication rate in combination with host physiology. However, there was a marked difference in CI between growth in blood and during infection for the ΔSp0750 mutant strain, whereas other mutant strains with reduced CIs in blood only had weakly reduced CIs during infection. These data perhaps indicate that the loss of virulence associated with mutation of LivJHMGF may be more complex than simple impaired growth under physiological conditions. The impaired CI of the Δ*livHMGF* strain compared to the wild-type strain when cultured in blood was not improved by the addition of exogenous BCAAs. This would be the expected result if LivJHMGF is a BCAA ABC transporter and there are no alternative *S. pneumoniae* low-affinity BCAA transporter systems that could compensate for a BCAA transport defect when BCAAs are present in high concentrations in the environment. Genes from both the *livJHMGF* and the Sp0445-50 operons have increased expression in cerebrospinal fluid in a rabbit model of meningitis (28) and are under the negative control of the transcriptional regulator CodY (16), but the relationship

between CodY repression, in vitro and in vivo expression of *livJHMGF*, and BCAA transport requires further investigation.

To conclude, we have investigated whether a range of *S. pneumoniae* ABC transporters affect virulence and identified that six of the nine investigated had some effect on the pathogenesis of infection compared to the wild-type strain using competitive infection experiments. The effects on virulence were generally weak, probably due to redundancy compensating for loss of function of individual ABC transporters. The functions of the majority of the ABC transporters affecting virulence were not clear, but we have characterized one encoded by *livJHMGF* in more detail and confirmed that it encodes a BCAA ABC transporter. Further investigation is required to identify the substrates of the remaining ABC transporters and to define why BCAA uptake is important for *S. pneumoniae* virulence.

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